REMARKS

The Office Action

Claims 16-19, 21-29, 44-46, and 48-118 are pending. Claims 44-50 and 117-118 are under consideration. All claims stand rejected for being anticipated by or for obviousness over Spence et al. (U.S. Publication No. 2002/0005354; hereafter "Spence").

Interview

Applicants thank the Examiner and her supervisor for the interview on March 27, 2007, in which the Office agreed to withdraw the present rejections over Spence. Applicants also present clarifying amendments requested by the Office.

Claim Amendments

All previously pending claims have either been cancelled or depend from claim 44 and therefore have unity of invention with claim 44. New claim 121 is identical to claim 44 except that the order of steps (i) and (ii) is reversed; thus, claim 121 and its dependent claims also have unity of invention with claim 44. The reference to gaps in claims 44 and 121 is supported in the specification on page 32, lines 5-6.

Claims 70 and 81 have been amended to delete steps recited in claim 44. Support for the amendment to claims 16 and 17 is found, for example, on page 3, line 2 to page 4, line 19 and page 35, lines 1-19. Claims 24, 25, 27, 71-73, 77, 80, 82, and 86 have been

amended for consistency with the claim from which each depends. New claims 121 and 124-133 find support in claims 44, 46, 72 – 76, and 86. New claims 119-120 and 122-123 find support in the specification on page 33, lines 22-29.

Spence

As discussed in person with the Examiner, Applicants traverse the rejection over Spence as either anticipating or rendering obvious the instant claims. §§ 102 and 103 require, at a minimum, that, to render a claim unpatentable, the prior art must teach or suggest all the limitations of the claims (M.P.E.P. §§ 2131 and 2143). Spence does not meet this standard as applied to the instant claims.

In support of the rejection, the Office has relied on paragraphs [0019], [0054], [0078], [0081], and [0082] (Action pages 4-5). After reviewing these paragraphs, Applicants submit that the Office had inadvertently mischaracterized the reference for the following reasons.

Claim 44 (as amended), from which all other claims depend, recites:

> 44. A method of producing a cell population enriched in a first type of cell larger than an adult, enucleated red blood cell, said method comprising the steps of subjecting a blood sample to (i) separation comprising contact with a microfluidic device comprising obstacles separated by gaps, so that adult, enucleated red blood cells and cells smaller than adult, enucleated red blood cells are directed in one direction and cells larger than adult, enucleated red blood cells are directed in a second direction to produce a first sample enriched in said cells larger than adult, enucleated red blood cells, and (ii) separation comprising contacting said first sample with a microfluidic device comprising obstacles that preferentially bind said first type of cell in

said first sample, thereby producing a population enriched in said first cell type.

Thus, claim 44 requires a method including at least two steps: a size-based separation step (i.e., (i)), and a binding-based separation step (i.e., (ii)). Each of these steps further employs a plurality of obstacles separated by gaps. Spence, in contrast, teaches neither the size-based separation step of (i) nor the binding-based separation step of (ii).

Spence is directed in general to a flow sorting device that separates cells flowing through the device based on a detectable signal (Abstract). Key to the technique of Spence is the detection of a property of each cell as it flows through a detection window in the device. As Spence describes, "[c]ells are diverted into one or another outlet channel based on a predetermined characteristic that is evaluated as each cell passes through the detection region." (page 2, [0012]). Spence further describes the device employed as including a main channel into which cells to be sorted are introduced (page 4, [0045]), a branch point (i.e., a junction) in which the direction of flow of cells can be altered (page 5, [0047]), and branch channels into one of which each cell is directed (page 5, [0048]).

Regarding step (i) of claim 44, requiring separation using a device comprising obstacles, the only channel having a plurality of obstacles described in Spence is shown in Figure 6. These obstacles are, unlike in claim 44, notably not disposed in the portions of the device required to effect flow separation, i.e., the main channel, the branch point (i.e., the junction), or the branch channels. The discussion of the device depicted in Figure 6

of Spence in paragraphs [0142] and [0143] (page 15) refers to a published scientific article, Chou et al. *Proc. Natl. Acad. Sci. USA* 1999, 96:11-13 (copy enclosed). The same device depicted in Figure 6 of Spence is shown in Fig. 1 of Chou, and the figure caption for Fig. 1 clearly states that the purpose of the obstacles is to "prop up the large channels and prevent bowing" (Chou, page 12). Furthermore, Spence examines each cell individually as it passes through the detection window and is then sorted (pages 1-2, [0009]), and there is no need for, and therefore no suggestion of, any separation of cells based on size – or indeed any other characteristic – prior to passing through the detection window.

In contrast to the teachings of Spence, the obstacles recited in claim 44 are essential for cell enrichment. Indeed, it is the differential interaction of the suspended, differently sized cells with these obstacles that results in enrichment. For example, in one embodiment of the invention, rows of obstacles are employed that only allow passage of cells below a certain size (page 32, lines 1-14, page 32, line 26 – page 33, line 5, and Figures 24A-24B). Without the obstacles, there would be no enrichment in step (i); in contrast, a lack of obstacles would not affect the fundamental operation of the methods disclosed in Spence. Thus, Spence provides no teaching or suggestion of the use of a plurality of obstacles in the separation of cells based on size, as instantly claimed.

With respect to step (ii), the Office has asserted that Spence inherently teaches binding of cells. Cells do not, however, inherently bind selectively to the surfaces of the

devices described by Spence. Indeed, Spence teaches the opposite: "To prevent the cells from adhering to the sides of the channels, the channels ... may have a coating which minimizes cell adhesion." (page 6, [0062]) In addition, Spence repeatedly refers to flows of cells through the device. For example, Spence defines a "flow" on page 4, [0041] as "movement of cells through a device or in a method of the invention." Moreover, Spence describes the "main channels," discussed above, as "a channel ... which permits the flow of cells past a detection region..." at page 4, [0045]. Flowing cells are by definition not bound to any surface. Binding of cells to the surfaces of the channels in Spence would also be highly undesirable because the purpose of the Spence device is to sort cells while they flow through the device. Applicants also note that even if cells did adhere to surfaces (and no evidence indicates that they do), claim 44 requires the preferential binding of a first cell type. Non-specific binding of cells to a surface is not preferential, and the Office has provided no evidence or reasoning why non-specific binding would suggest the preferential binding instantly claimed. Finally, step (ii) requires the binding of cells to obstacles in a microfluidic device, and, as discussed above, the only obstacles disclosed by Spence are in the context of preventing collapse of a channel that is not an integral part of Spence's invention. For all these reasons, Spence does not teach or suggest preferential binding of cells to obstacles in a microfluidic device, as recited in step (ii).

As the cited reference fails to teach or suggest either of the steps required by the instant claims, the rejection may be withdrawn. In addition, as the Office has failed to establish a *prima facie* case of anticipation or obviousness for the independent claim, it is unnecessary to address the Office's arguments with respect to the dependent claims. Applicants reserve the right to do so in the future, if necessary.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested. Enclosed is a petition to extend the period for reply for one month, to and including May 5, 2007. If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: April 11, 2007

Paul T. Clark
Reg. No. 30,162

J. Cooper McDonald, Ph.D.
Reg. No. 52,011

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 11–13, January 1999
Applied Physical Sciences, Biophysics

A microfabricated device for sizing and sorting DNA molecules

HOU-PU CHOU, CHARLES SPENCE, AXEL SCHERER, AND STEPHEN QUAKE*

Department of Applied Physics, California Institute of Technology, Pasadena, CA 91125

Communicated by John D. Baldeschwieler, California Institute of Technology, Pasadena, CA, October 26, 1998 (received for review August 28, 1998)

ABSTRACT **We have demonstrated a microfabricated single-molecule DNA sizing device. This device does not depend on mobility to measure molecule size, is 100 times faster than pulsed-field gel electrophoresis, and has a resolution that improves with increasing DNA length. It also requires a million times less sample than pulsed-field gel electrophoresis and has comparable resolution for large molecules. Here we describe the fabrication and use of the single-molecule DNA sizing device for sizing and sorting DNA restriction digests and ladders spanning 2–200 kbp.**

Many assays in biology require measurement of the length distribution of DNA molecules in a heterogeneous solution. This measurement is commonly done with gel electrophoresis; the molecules are separated by mobility, from which the lengths are inferred. This method is powerful, yet has some drawbacks. For medium to large DNA molecules the resolution is limited to approximately 10%. Gel electrophoresis is time consuming. It generally takes at least an hour to run the gel, not including the setup time to cast the gel. Furthermore, for large molecules the procedure fails. This problem has been alleviated to some extent by the development of pulsed-field gel electrophoresis (1), but running times can be days.

With the development of high affinity intercalating DNA stains (2), it has become possible to directly measure the length of single molecules by quantitating fluorescence. The amount of intercalated dye is proportional to the length of the molecule, so measuring the total fluorescent intensity from a single molecule gives a direct measurement of its length. This method in principle allows the measurement of extremely long DNA molecules because the signal increases with the length of the molecule. This technique has been used with traditional methods of flow cytometry to measure length distributions of DNA molecules (3, 4). Other groups have imaged restriction enzymes digesting extended single DNA molecules for "optical mapping" (5, 6).

We have developed microfabricated devices to size and sort microscopic objects based on measurement of fluorescent properties. The devices have a network of microfluidic channels and are fabricated from a silicone elastomer by using a replica technique (7). Master molds are made from silicon wafers by using standard micromachining techniques. Because the molds can be reused indefinitely, this method of fabrication allows economical mass production of the devices. The devices were patterned as shown in Fig. 1. This fabrication technique is one of a new set of technologies known as soft lithography. Previous work has demonstrated that elastomers can replicate gratings and other test patterns with high (≈50 nm) resolution and fidelity (8, 9). Although some groups have made large (≈30 μm) elastomer structures for capillary electrophoresis (10), there is only one other example of a micron scale fluidic network with the elastomer (11).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Device Fabrication. Negative master devices were fabricated in silicon and used as molds for the silicone elastomer. Contact photolithography was used to pattern the oxide surface of a silicon wafer, which then was etched by reactive ion etch (RIE) with a C_2F_6/CHF_3 gas mixture. A 3-min O_2 RIE was used to remove fluorocarbon polymer residue on the silicon surface. The oxide then was used as a mask for the silicon underneath, which was etched with KOH. The silicone elastomer (General Electric RTV 615) components were mixed together and pumped in an evacuated chamber for 30 min to remove air bubbles. The liquid elastomer then was poured on the mold and cured in an oven at 90°C for 2 hr. After this procedure, the devices could be peeled from the silicon master and would bond hermetically to glass. Number 1 coverslips were used to seal the devices.

The elastomer is naturally hydrophobic, preventing aqueous solution from entering the channels. The surfaces of the devices were modified by soaking in dilute HCl (pH 2.7, 0.01% in water) for 40 min at 43°C. After this treatment, the devices were hydrophilic, and aqueous solution would enter easily by capillary action. The devices could be cleaned and reused several times if desired. All of the data for this paper were taken with the same device. We have found that the geometry of the device does not affect reproducibility of the results; data taken from a device with slightly different channel dimensions were comparable. Results from run to run and day to day are highly reproducible; the histograms can be overlaid without adjusting any parameters and there is no need to recalibrate the apparatus.

Sample wells were created during the fabrication process by gluing small aluminum cylinders to the silicon wafer molds. The final elastomer devices were attached to a coverslip in such a way that the sample wells were only partially covered, which allowed access with a pipette tip for introduction of the sample and also provided convenient electrode insertion. The flow rate was determined by a balance between capillary action within the channels and electro-osmotic flow.

Experimental Apparatus. A 10-mW air-cooled argon ion laser (Uniphase, San Jose, CA) emitting at 488 nm was used for fluorescent excitation. The laser was focused through a 60× 1.4 NA oil immersion objective, which also was used to collect the emitted fluorescence, on an upright microscope (Olympus BH-2, New Hyde Park, NY). Auxiliary lenses were used to adjust the focused spot to a full width half maximum of 30 μm. The large spot size was chosen to give uniform excitation across the width of the channel. The quality and uniformity of the spot was evaluated by imaging a thin layer of fluorescein in solution with a charge-coupled device camera. The image was digitized and evaluated for symmetry and Gaussian shape. Dielectric filters were used to filter laser tube fluorescence (CVI 488-nm line filter, CVI Laser, Albuquerque, NM) and to reduce background and scattered light from

Abbreviation: SMS, single-molecule DNA sizing.

*To whom reprint requests should be addressed at: Department of Applied Physics, California Institute of Technology, MS 128-95, Pasadena, CA 91125. e-mail: quake@caltech.edu.

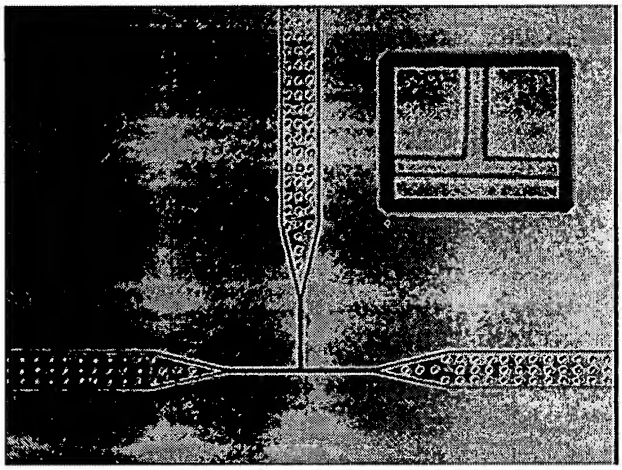

FIG. 1. Optical micrograph of T-channel device. The large channels have lateral dimensions of 100 μm, which narrow down to 5 μm at the T junction. The depth of the channels is 3 μm. In early prototypes, we found that because of the large aspect ratio (100 μm in width by 3 μm in depth), some of the elastomer channels would bow and pinch off by sealing directly to the glass. This problem was remedied in later versions by adding support pillars to the mask that would prop up the large channels and prevent bowing. (*Inset*) Magnified view of T junction. The channels are 5 μm wide at this point. Note the high fidelity of the elastomer replica.

the emitted fluorescence (Chroma D535/50M, Chroma Technology, Brattleboro, VT). A dichroic filter was used to introduce the laser light into the optical train (Chroma 500 DCLP).

Fluorescence was imaged onto a 5-mm avalanche photodiode detector (Advanced Photonix, Camarillo, CA). The detector was cooled to −40° with a two-stage thermoelectric cooler (ITI 6320/157/040C, Chelmsford, MA), which reduced the dark current of the detector from 50 nA to 90 pA. The detector was reverse-biased at 2,450 V, giving a gain of 500. A transimpedance amplifier (Burr Brown OP128, Tucson, AZ) converted the photocurrent to a voltage at a gain of 100 mV/nA. A second stage amplifier provided additional voltage gain of 10. The signal was low-pass-filtered at 1.6 kHz and digitized at 5 kHz by a National Instruments (Austin, TX) Lab PC1200 board on a personal computer running LABVIEW.

The depth of focus of the microscope was checked by centering a 1-μm fluorescent bead in the laser beam. The detector output as a function of focal distance shows that the signal is essentially flat over a depth of 5 μm. The depth of the device channels was chosen to be 3 μm so that the DNA molecules always remained in the plane of focus of the microscope.

Sample Preparation. Lambda phage DNA (GIBCO) was either digested with *Hin*dIII (GIBCO) or ligated with T4 ligase (New England Biolabs), then was diluted in buffer (Tris-EDTA, pH 6.8 with 5 mM NaCl) and stained with the intercalating dye YOYO-1 (Molecular Probes) at a stoichiometry of one dye molecule per 4 bp. Single molecules of DNA gave measurable pulses whose height corresponded to the length of the molecule. Pulses were collected in large batches and then analyzed off-line with custom software written for peak detection. One of the advantages of this device is the simplicity of the data processing: no fitting is needed. The analog signal from the avalanche photodiode is digitally low-pass-filtered (cutoff ≈300 Hz), and peak heights are extracted with a thresholding algorithm. The analysis is extremely efficient to implement in real time and can be done for DNA sorting applications, as described below.

RESULTS

To test the utility of these devices for screening restriction digests, we analyzed a *Hin*dIII digest of λ DNA. A solution

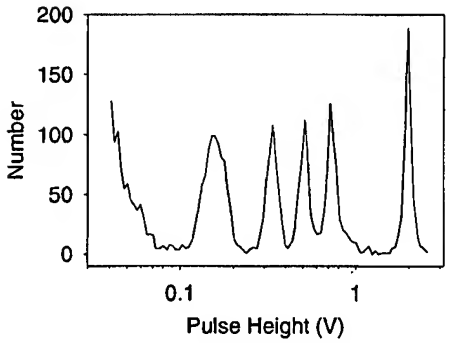

FIG. 2. Histogram of *Hin*dIII digest of λ DNA. The peaks represent (from right to left) fragments of length 23 kbp, 9 kbp, 6 kbp, 4 kbp, and an unresolved combination of 2 kbp and 2.3 kbp.

containing the stained digest was introduced to the device, and fluorescence was collected with a microscope and monitored with an avalanche photodiode. Fig. 2 shows a histogram of the observed peak heights. After collection of data for 10 min, the major fragments are clearly resolved. The 2- and 2.3-kbp pieces are not resolved from each other, but are well above the noise floor of the device. For larger fragments, the resolution is on the order of 5% and improves with the length of the molecule (Fig. 3). A notable feature of this method is that it requires very small amounts of sample: 28 femtograms of DNA were analyzed, about 3,000 molecules.

The detection volume for the single-molecule DNA sizing (SMS) devices is 375 femtoliters, more than an order of magnitude smaller than what has been achieved so far with flow cytometry. This small detection volume reduces the background signal proportionately. Furthermore, the geometry of the microfabricated devices allows the use of a high numerical aperture objective for efficient collection of the fluorescent light. DNA fragments of size 2 kbp, containing ≈500 fluorophores, were easily measured. The noise floor of the histogram for the *Hin*dIII digest indicates that the current sensitivity of the device is approximately 1 kbp.

To determine the upper length limits of analysis in the device, we analyzed λ DNA ladders in the devices and have been able to detect molecules of up to 200 kbp (Fig. 4). That limit is determined solely by the geometry of the optical setup and does not represent an inherent limitation of the technology. As with the *Hin*dIII digest, about 3,000 molecules were analyzed in 10 min.

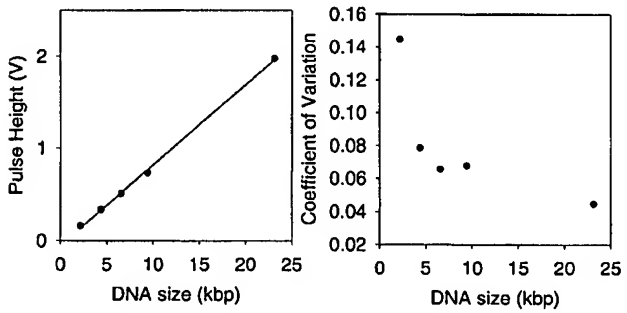

FIG. 3. Precision and resolution of *Hin*dIII digest. The histogram of Fig. 2 was fit with 5 Gaussians to estimate the precision and resolution of the measurement. (*Left*) The known sizes of the restriction fragments are compared with the fitted peak locations. The measurements are linear with a precision of a few percent. (*Right*) The widths of the peaks determine the resolution of the measurement. The coefficient of variation is the peak's SD divided by its height and is an indication of the fractional resolution. Resolution improves with longer molecules. In both graphs, the error bars are smaller than the size of the data point symbols.

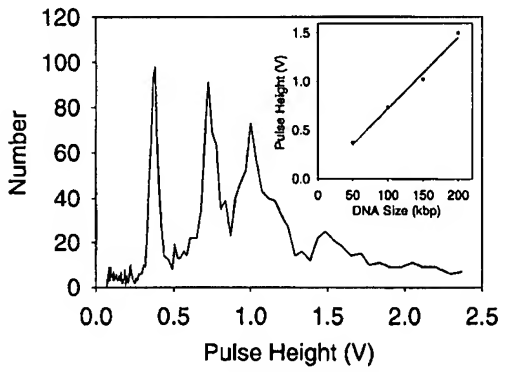

FIG. 4. Analysis of λ ladder. To test the upper length limit of the device, a λ ladder was analyzed. Peaks corresponding to 50, 100, 150, and 200 kbp can clearly be resolved. (*Inset*) The peak height measurement is linear even out to 200 kbp.

The microfabricated devices have several advantages over macroscopic systems, including design flexibility, size, cost, and sensitivity. An important example of this flexibility is that one can actively sort molecules in the microfabricated devices. By inserting electrodes in the sample wells and manipulating the DNA with electric fields, we have shown that molecules can be sent down one channel or the other at will. Speeds of 125 μm/sec were attained with an electric field of 20 V/cm. The response time of the DNA to a rapidly switching potential was faster than 30 msec, the video data acquisition rate. Thus, after detection, if the molecule passes a given criterion, a computer or circuit can be used to control the destiny of the molecule. The T channels can be cascaded to give an arbitrary number of bins in which the molecules can be collected. In contrast, the hydrodynamic focusing methods used in flow cytometry do not permit easy manipulation of the sample after detection.

DISCUSSION

We have shown that the SMS devices can be used for rapid, efficient sizing of DNA molecules ranging from 2 to 200 kbp. The operating time of 10 min is independent of the size of the DNA molecules being sorted and represents a significant improvement over gel electrophoresis. Furthermore, the devices require only tens of femtograms of DNA to operate, making it is possible to envision future applications in which SMS devices obviate the need for PCR.

By directly measuring length via fluorescent dye intercalation, SMS devices allow an absolute measurement of length which eliminates the need for sizing standard in each run. Finally, for gel electrophoresis the resolution decreases as the molecules being analyzed become longer. SMS has the opposite property: the longer the molecules, the better the signal to noise, and the fewer fluctuations because of the statistics of dye binding (Fig. 3). For the longest molecules in the lambda ladder (Fig. 4), the SMS resolution begins to degrade as a result of the optical setup: the molecules become bigger (or longer) than the region illuminated by the laser beam used to excite fluorescence. This problem can be easily remedied in the future, as applications require. The ultimate sizing limit probably will be determined by the ability of DNA molecules to survive fluid shear forces in the device.

There are several possible direct applications for SMS DNA sizing. Restriction fragment length polymorphism analysis and genetic mapping are amenable to analysis in these devices. Another important application is in the Human Genome Project. There has been a proposal to use bacterial artificial chromosome (BAC) libraries for human genome sequencing (12). This proposal requires restriction digest fingerprinting of 600,000 BAC clones, a slow and expensive task with current technology. SMS devices are capable of rapid, cheap restriction fingerprinting and mapping of BAC clones, which have a typical size of 200 kbp.

Because the devices are microfabricated, additional features could be added directly to the chip. It is easy to construct many devices in parallel on a chip, allowing rapid multiplex analysis of samples. One can envision future SMS devices with cascaded T channels capable of sorting fragments according to size and then recutting the individual fragments with different enzymes for ordered mapping, something that is not possible with electrophoretic or optical mapping techniques. Furthermore, because the detection system is all solid state and does not in principle require imaging optics, it should be possible to build an integrated SMS device with semiconductor laser excitation and detection on a single chip.

We thank Wayne Volkmuth for helpful discussions and contributions in the early stages of this work. This work was supported in part by the Powell Foundation and National Institutes of Health Grant HG01641-01.

1. Burmeister, M. & Ulanovsky, L., eds. (1992) *Pulsed-Field Gel Electrophoresis: Protocols, Methods, and Theories* (Humana, Totowa, NJ).
2. Haugland, R. P. (1996) *Handbook of Fluorescent Probes and Research Chemicals* (Molecular Probes, Eugene, OR).
3. Castro, A., Fairfield, F. R. & Schera, E. B. (1993) *Anal. Chem.* **65**, 849–852.
4. Huang, Z., Petty, J. T., Quinn, O. B., Longmire, J. L., Brown, N. C., Jett, J. H. & Keller, R. A. (1996) *Nucleic Acids Res.* **24**, 4202–4209.
5. Guo, X. H., Huff, E. J. & Schwartz, D. C. (1992) *Nature (London)* **359**, 783–784.
6. Cai, W. W., Jing, J. P., Irvin, B., Ohler, L., Rose, E., Shizuya, H., Kim, U. J., Simon, M., Anantharaman, T., Mishra, B. & Schwartz, D. C. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 3390–3395.
7. Carlson, R. H., Gabel, C. V., Chan, S. S., Austin, R. H., Brody, J. P. & Winkelman, J. W. (1997) *Phys. Rev. Lett.* **79**, 2149–2152.
8. Jackman, R. J., Wilbur, J. L. & Whitesides, G. M. (1995) *Science* **269**, 664–666.
9. Xia, Y. & Whitesides, G. M. (1998) *Angew. Chem. Int. Ed. Engl.* **37**, 550–575.
10. Effenhauser, C. S., Bruin, G. J. M., Paulus, A. & Ehrat, M. (1997) *Anal. Chem.* **69**, 3451–3457.
11. Delamarche, E., Bernard, A., Schmid, H., Michel, B. & Biebuyck, H. (1997) *Science* **276**, 779–781.
12. Venter, J. C., Smith, H. O. & Hood, L. (1996) *Nature (London)* **381**, 364–366.